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## **Genetic variants of methionine metabolism and DNA methylation**

Bleich, Stefan ; Semmler, Alexander ; Frieling, Helge ; Thumfart, L ; Muschler, Marc ; Hillemacher, Thomas ; Kornhuber, Johannes ; Kallweit, Ulf ; Simon, Matthias ; Linnebank, Michael

**Abstract:** AIM: Altered DNA methylation is associated with important and common pathologies such as cancer. The origin of altered DNA methylation is unknown. The methyl groups for DNA methylation are provided by methionine metabolism. This metabolism is characterized by a high interindividual variability, which is in part explained by genetic variants. **METHODS:** In a cohort of 313 individuals derived from a family-based study with index cases of cerebrovascular disease, we analyzed whether global methylation of leukocyte DNA was associated with age, gender, homocysteine plasma levels or functionally relevant genetic variants. **RESULTS:** We observed an association of the G-allele of the methionine synthase variant c.2756A>G (D919G) with global methylation (% methylation  $\pm$  1 SD, AA:  $41.3 \pm 14.9$ ; AG:  $36.4 \pm 18.2$ ; GG:  $30.8 \pm 16.9$ ;  $F = 4.799$ ;  $p = 0.009$ ). The methionine synthase variant c.2756A>G is associated with various types of cancer. **CONCLUSION:** Our data suggest that an impact on DNA methylation may contribute to the clinical relevance of the methionine synthase variant.

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## **Genetic variants of methionine metabolism and DNA methylation**

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Keywords: DNA methylation; homocysteine; methionine metabolism; single nucleotide

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polymorphism

## **Abstract**

Altered DNA methylation is associated with important and common pathologies such as cancer. The origin of altered DNA methylation is unknown. The methyl groups for DNA methylation are provided by methionine metabolism. This metabolism is characterized by a high inter-individual variability, which is in part explained by genetic variants. In a cohort of 313 individuals derived from a family-based study with index cases of cerebrovascular disease we analysed whether global methylation of leukocyte DNA was associated with age, gender, homocysteine plasma levels or functionally relevant genetic variants. We observed an association of the G-allele of the methionine synthase variant c.2756A>G (D919G) with global methylation (% methylation  $\pm$  1 standard deviation, AA: 41.3 $\pm$ 14.9; AG: 36.4 $\pm$ 18.2; GG: 30.8 $\pm$ 16.9; F=4.799; p=0.009). The methionine synthase variant c.2756A>G is associated with various types of cancer. Our data suggest that an impact on DNA methylation may contribute to the clinical relevance of the methionine synthase variant.

**Keywords:** DNA methylation; homocysteine; methionine metabolism; single nucleotide polymorphism

## **Introduction**

DNA methylation is a well-known epigenetic mechanism and associated with important and common pathologies such as cancer [1]. The origin of altered DNA methylation, however, remains unclear. Recently, a variety of inactivating mutations in genes controlling the epigenome have been described in whole genome sequencing of human cancers [2,3]. These mutations have the potential to disrupt DNA methylation patterns and therefore contribute to disease development as much as epigenetic silencing is able to cause the loss of function of genes and predisposes to genetic mutations, leading to an interplay between genetic mutations and epigenetic alterations. Several human cancers show mutations of epigenetic modifiers that presumably lead to altered DNA methylation. In acute myeloid leukemia DNA methyltransferase 3a (DNMT3A) mutations have been found to be associated with differences in global DNA methylation patterns [4], in colorectal cancer mutational inactivation of DNMT1 was described [5], and isocitrate dehydrogenase 1/2 (IDH 1/2) mutated gliomas show distinct epigenetic profiles, characterized by a high frequency of methylated CpG islands [6].

The human methionine metabolism is closely linked to DNA synthesis and epigenetics (Figure 1). Methionine is activated to S-adenosyl-methionine (SAM), which is the only and ubiquitous methyl donor for DNA methylation mediated by the DNA methyltransferases. Methionine metabolism is known to be influenced by several factors including genetic variants and nutrition. Some genetic variants of methionine metabolism have been reported to be associated with vascular disease, neurodegeneration or cancer [7]. It is therefore plausible to assume that some genetic variants of the methionine metabolism could influence DNA methylation. This study investigated whether genetic variations of methionine metabolism influence the global DNA methylation in sample of individuals of whom several genetic variations of methionine metabolism were already known [8].

## **Materials and Methods**

For this study we used a gDNA library consisting of 313 gDNA samples of German individuals of white Caucasian ancestry from an ongoing study investigating the inheritance of the wall thickness of the common carotid intima media thickness [8,9]. Of the 313 individuals, n= 85 were patients with vascular disease (mean age in years  $\pm$  1 standard deviation:  $63.4 \pm 8.9$ ; 22% female), n= 68 were spouses ( $62.0 \pm 8.7$  years; 79% female), and n= 160 were offspring ( $35.0 \pm 7.9$  years, 51% female) of these patients and spouses. All patients with clinical or laboratory signs of inflammation, including changes in complete blood count (CBC) and increases of C-reactive protein (CRP), were excluded from the study. All patients with laboratory signs of decreased kidney function were excluded from the study. Individuals were of Caucasian origin and lived in the area of Bonn, Germany. The patients with vascular disease were defined by an increase of the intima-media thickness of the common carotid artery [9]. Spouses and offspring had no increase of the intima-media thickness and no history of vascular events. For these 313 individuals, genotypes were known for the following genetic variants of methionine metabolism, i.e. CBS c.844\_855ins68 (change of transcript levels, Genbank Number S78267.1)[10], DHFR c.594+59del19bp (change of transcript levels, rs70991108)[11], MTHFR c.677C>T (A222V, rs1801133) [12] and c.1298A>C (E429A, rs1801131) [13], MTR c.2756A>G (D919G, rs1805087) [14], and reduced folate carrier 1 (RFC1) c.80G>A (R27H, rs1051266) [8,9,15]; Figure 1.

Plasma homocysteine levels were determined by fully automated particle-enhanced immunonephelometry with a BN II System (Dade Behring).

#### Measurement of DNA methylation

Total DNA was extracted from frozen EDTA-blood using QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Global methylation status was measured as previously described [16]. Briefly, 0.5-1  $\mu$ g of genomic DNA is digested overnight with a 10-fold excess of HpaII or MspI end nuclease according to manufacturer's protocol (New England Biolabs, Beverly,

MA). MspI cuts independently from methylation status at 5'-CCGG-3', while cutting of HpaII depends on the DNA-methylation status. The single nucleotide extension reaction is performed in a 25 µl reaction mixture containing 2 Units of polymerase (Genecraft: Supratherm, Lüdinghausen, Germany), polymerase-buffer (Genecraft) and 0.4 mM Cy5-labelled dCTP (GE Healthcare Europe, Munich, Germany), and incubated at 56° for 1 h on an I-Cycler (Biorad, Hercules, CA) to allow filling of overlapping ends with dCTPs, then placed on ice. Free dCTPs are separated by QIAquick Nucleotide removal kit (Qiagen) and fluorescently labelled DNA is eluted by 100µl of H<sub>2</sub>O. Fluorescence is measured on a Molecular Imager FX (Biorad) and quotients of 1-(HpaII/MspI) are calculated from 3 independent experiments [16].

#### Statistical analysis

The Kolmogorov-Smirnov Test revealed normal distribution of all data analysed. Deviation from Hardy-Weinberg-Equilibrium was tested for all genotypes studied using the online tool of the Institute for Human Genetics, Munich, accessible at <http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>. Mixed linear models (using the restricted estimated maximum likelihood method) were performed to evaluate the impact of the analysed genotypes on global DNA methylation. Akaike's information criterion (AIC) was used to assess the best fitting model. To cope with the family structure within our sample, we computed families as repeated measurements applying a scaled identity covariance structure. Linear regression analysis was used to test associations between the covariates homocysteine, age, smoking, and all tested genotypes and global DNA methylation as dependent variable. Comparison of the global DNA methylation in different genotypes of the same genetic variant was made using one-way analysis of variance (ANOVA).

P-values of less than 0.05 (two-tailed) were considered to indicate statistical significance.

Data were analyzed employing the statistical package for the social sciences (SPSS<sup>TM</sup> for

Windows 14.0; SPSS Inc., Chicago, IL).

## Results

Linear regression analysis revealed no significant influence of age ( $B=-0.42$ ;  $P=0.469$ ) and homocysteine ( $B=0.194$ ;  $P=0.392$ ) on global DNA methylation. Neither did gender (t-Test:  $T=0.007$ ;  $df=311$ ;  $P=0.995$ ) nor smoking status ( $T=0.302$ ;  $df=296$ ;  $P=0.763$ ).

All genotypes studied did not show deviation from Hardy-Weinberg-Equilibrium with the exception of DHFR19. Interestingly, this deviation was only driven by the offspring group in the study population. As we did not observe significant differences in genotype distribution between the three groups (cases, offspring, spouses), we decided to include DHFR into the subsequent analyses. Table 2 provides the investigated genotypes and their association with global DNA methylation. F and P-values were derived from the parameter estimates and corrected for multiple testing using Bonferroni's procedure. We analyzed whether the proportion of variance of DNA methylation derived by the single genotypes was significant.

As shown in Table 2, the MTR c.2756A>G genotype was associated with global DNA methylation showing an allele-dosage effect with a higher methylation associated with the wild-type A-allele. This association was only significant for the total sample size, but not, if patients, spouses and offspring were analysed separately. We did not observe any further significant results for all other investigated genotypes (Table 2). We tested our model in a general linear analysis and observed that the MTR c.2756A>G genotype ( $p=0.048$ ; 50.88% of variance), but not age, sex, family status or any interaction between factors and covariates was associated with global DNA methylation. Homocysteine plasma levels were not associated with MTR alleles (ANOVA:  $F=0.87$ ;  $p=0.419$ ). Homocysteine plasma levels did not correlate with methylation (Pearson's correlation:  $-0.033$ ;  $p=0.555$ ).

## Discussion

This study shows that the G-allele of MTR c.2756A>G (D919G) is associated with reduced global DNA-methylation.

The genotype frequencies of the current study are within the range reported in other study populations for CBS c.844\_855ins68 [10,17], DHFR c.594+59del19bp [11,18], MTHFR c.677C>T [12,19] and c.1298A>C [13,20], RFC1 c.80G>A [15], and MTR c.2756A>G [14,21]. This shows that all genotype frequencies reported in the current study are representative and are not due to the current study population or patient selection.

The mechanism how MTR c.2756A>G leads to reduced global DNA-methylation remains speculative as the biological effect of MTR c.2756A>G is unclear. *In vitro* analyses of the variant enzyme activity are lacking, and associations of the different alleles with homocysteine plasma levels are controverse [22,23]. Paz et al. found that in tumors occurring in individuals who carried 2756GG showed a lower frequency of CpG island hypermethylation in tumor suppressor genes [24]. In the population of our study we did not detect an association between the MTR genotype and homocysteine plasma levels or between homocysteine levels and DNA methylation. This suggests that the MTR variant might have an effect on DNA methylation independent from effects on homocysteine levels, alternatively homocysteine levels might additionally be controlled by altered flux through the transsulfuration or the alternative remethylation pathway based on the enzyme betaine-homocysteine methyltransferase [25].

The G-allele of MTR c.2756A>G has already been reported to decrease the susceptibility to various cancers [26-31]. The observed effect of the G-allele of the MTR c.2756A>G variant on DNA methylation suggests that the protective effect of this variant on several types of cancer may be mediated decreased CpG island methylation, because hypermethylation of DNA repair and tumor suppressor genes can result in the inactivation of these genes resulting



in increased levels of genetic damage, predisposing cells to later genetic instability which then contributes to tumor progression [32]. For example, the tumour suppressor genes Von Hippel–Lindau (*VHL*) in renal cancer and *CDKN2A* in several tumour types are DNA hypermethylated [33,34]. This may also explain why the G-allele of MTR c.2756A>G has been described to be associated with longevity [35].

A recent paper of Weiner et al, described that the homozygous genotype of MTR c.2756GG is associated with increased global DNA methylation when compared to homozygous carriers of the MTRc.2756AA wildtype in their study population of healthy Caucasian Russian blood donors. In addition to that they showed a significant decrease of global DNA methylation in homozygous MTHFR c.677TT carries in comparison to probands with the MTHFR c.677CC genotype [36]. The difference between this study and the current data might be explained by differences in populations and other factors influencing DNA methylation such as folate levels, alcohol consumption, and smoking [37,38].

Finally, it is tempting to speculate about possible clinical implications of the data presented. MTR activity depends on folate and vitamin B12 [25], which might provide a link of nutritional factors, epigenetics and cancer development [39]. Additional studies on the functional consequences of the MTR c.2756A>G polymorphism and of folate and vitamin B12 on the DNA methylation patterns are warranted.

Our results are limited due to different factors: First, we did not analyze a population based cohort, but families with at least one person suffering from vascular disease. However, in all subgroups defined by family status, results tended in the same direction making it unlikely that the overall effect is driven by a specific effect in vascular patients only. Second, we did not investigate genetically independent subjects, but family members. We tried to control for this influence in the mixed models by treating family members as linked samples and modeling the covariance structure accordingly. Further, the power of the study might not have been sufficient to detect small effects of genotypes on DNA methylation. In a study of 384

healthy Japanese women, the amount of dietary folate intake, but not MTR c.2756A>G, was associated with global methylation of leukocyte levels [40]. Methionine metabolism is strongly influenced by folate and vitamin B12 intake and blood levels, and such parameters were not measured in our study. Thus, we cannot decide whether differences in these parameters might have contributed to the discrepancy of the results between the Japanese and our study population.

#### Future perspective

Global DNA methylation is an important epigenetic mechanism and associated with clinically important diseases such as cancer. This study shows that the G-allele of MTR c.2756A>G (D919G) is associated with reduced global DNA-methylation. Future studies have to describe the further mechanisms leading to altered DNA methylation including other genetic variations, but also environmental influences like nutrition or medications. Probably there is a complex interplay between all factors leading to altered DNA methylation and precise characterization of the study population is essential to achieve meaningful results.

#### Executive Summary

- The origin of altered DNA methylation is unknown.
- In a cohort of 313 individuals we observed an association of the G-allele of the methionine synthase variant c.2756A>G (D919G) with global methylation.
- Our data suggest that an impact on DNA methylation may contribute to the clinical relevance of the methionine synthase variant.

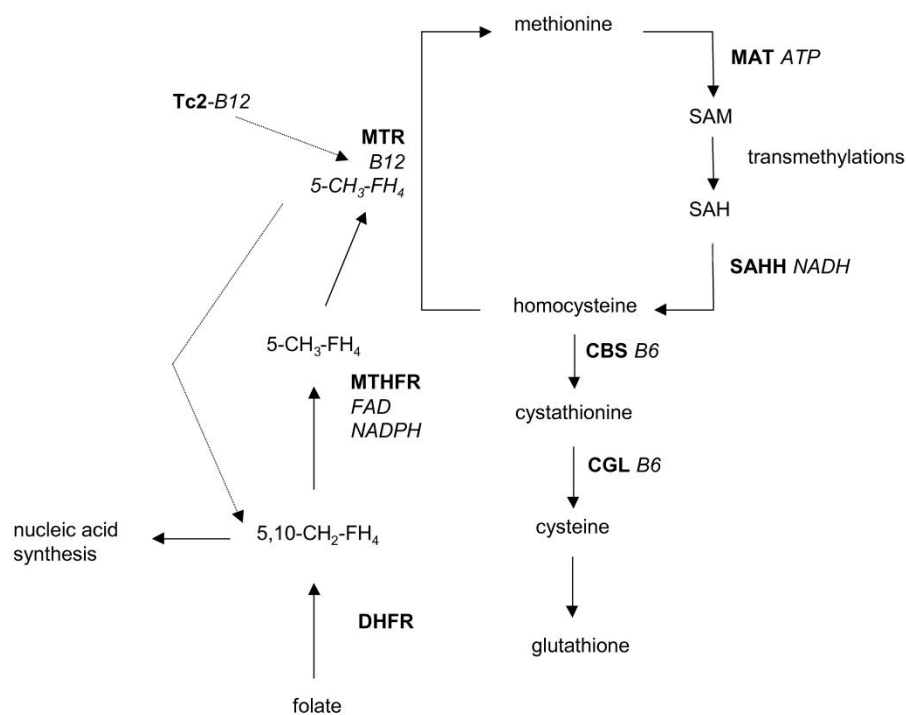
**Table 1. Polymorphisms and global leukocyte CpG island methylation**

Variant rs number/Genbank No.	genotype number of individuals (%) global DNA methylation			ANOVA	HWE (Pearson's)
CBS c.844_855ins68 S78267.1	del/del: 274 (87.5%) 39.3±16.2	del/ins: 38 (12.1%) 37.2±18.8	ins/ins: 1 (0.3%) 34.4	F=1.237 p=0.292	F=-0.015 p=0.83
DHFR c.594+59del19bp NM_000791.3	del/del: 70 (22.4%) 40.0±16.0	del/ins: 134 (42.8%) 38.9±16.7	ins/ins: 109 (34.8%) 38.0±16.9	F=0.313 p=0.732	F=0.13 p=0.02 <sup>#</sup>
MTHFR c.677C>T rs1801133	CC: 151 (48.2%) 38.6±15.9	CT: 139 (44.4%) 39.6±17.4	TT: 23 (7.3%) 36.3±16.0	F=0.396 p=0.673	F=-0.062 p=0.27
MTHFR c.1298A>C rs1801131	AA: 151 (48.2%) 39.4±16.1	AC: 136 (43.4%) 38.8±17.5	CC: 26 (8.3%) 36.1±14.4	F=0.742 p=0.477	F=-0.029 p=0.60
RFC1 c.80G>A rs1051266	GG: 102 (32.6%) 40.6±19.1	AG: 149 (47.6%) 39.7±16.1	AA: 62 (19.8%) 36.6±15.4	F=1.534 p=0.217	F=0.037 p=0.51
MTR c.2756A>G rs1805087	AA: 184 (58.8%) 41.3±14.9	AG: 112 (35.8%) 36.4±18.2	GG: 17 (5.4%) 30.8±16.9	F=4.799 p=0.009*	F=-0.012 p=0.83
MTR c.2756A>G Patients only	AA: 50 (58.8%) 33.5±21.3	AG: 30 (35.3%) 32.9±24.0	GG: 5 (5.9%) 16.1±38.9	F=1.279 p=0.284	F=-0.012 p=0.91
MTR c.2756A>G Spouses only	AA: 42 (61.8%) 38.3±19.9	AG: 22 (32.4%) 34.6±21.8	GG: 4 (5.9%) 27.0±21.5	F=1.599 p=0.205	F=0.059 p=0.63
MTR c.2756A>G Offspring only	AA: 92 (57.5%) 38.3±19.9	AG: 60 (37.5%) 34.6±21.8	GG: 8 (5.0%) 27.0±21.5	F=1.599 p=0.205	F=-0.047 p=0.60

Table 1: All tested genetic variants of the methionine metabolism and their association with global DNA methylation. The MTR variant c.2756A>G (allele-dosage effect with the wildtype A-allele) was significantly associated with global DNA methylation (\*). CBS (cystathionine beta-synthase), DHFR (dihydrofolate reductase), MTHFR (5,10-

methylenetetrahydrofolate reductase), MTR (5-methyltetrahydrofolate-homocysteine S-methyltransferase), RFC (reduced folate carrier).

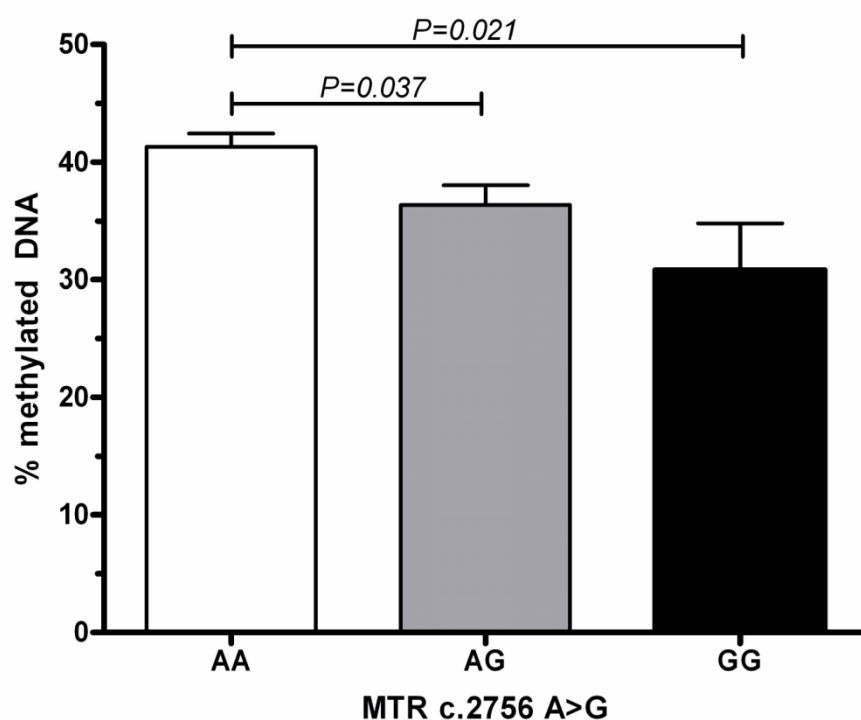
**Figure 1. Methionine-homocysteine metabolism**



**Figure 1:** In human methionine metabolism methionine becomes activated to S-adenosylmethionine (SAM) via methionine adenosyltransferase (MAT). SAM is an ubiquitous methyl group donor, e.g., for the synthesis of biogenic amines and DNA methylation. Degradation product of SAM is S-adenosylhomocysteine (SAH), which is hydrolyzed to homocysteine per homocysteine hydrolase (SAHH). Hcys can either be transsulfurated to cystathionine and cysteine via vitamin B6-dependent cystathionine beta-synthase (CBS) and cystathionine gamma lyase (CGL), or alternatively, homocysteine can be remethylated to methionine. This is done by 5-methyltetrahydrofolate-homocysteine S-

methyltransferase (MTR, also called methionine synthase), which needs a derivate of vitamin B12 (methylcobalamin) as well as a derivate of folate (5-methyltetrahydrofolate; 5-MTHF) as cofactors. Vitamin B12 is transported by transcobalamin 2 (Tc2), and 5-methyltetrahydrofolate is synthesized by 5,10-methylenetetrahydrofolate reductase (MTHFR) from 5,10-methylenetetrahydrofolate (5,10-MTHF), which is synthesized from folate by dihydrofolate reductase (DHFR) in two subsequent steps.

**Figure 2. Impact of MTR c.2756A>G on global DNA methylation**



**Figure 2:** MTR c.2756 A>G genotypes and global DNA methylation. P values given are derived from the all pairwise comparison of the estimated marginal means and are corrected according to Bonferroni.

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\*\* very concise review about the interplay between genetic and epigenetic variations in cancer

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